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ARTICLE

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DNA-induced endocytosis upon local microinjection to giant unilamellar cationic vesicles

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Abstract We suggest a novel approach for direct optical microscopy observation of DNA interaction with the bilayers of giant cationic liposomes. Giant unilamellar vesicles, about 100 µm in diameter, made of phosphatidylcholines and up to 33 mol% of the natural bioactive cationic amphiphile sphingosine, were obtained by electroformation. "Short" DNAs (oligonucleotide 21b and calf thymus 250 bp) were locally injected by micropipette to a part of the giant unilamellar vesicle (GUV) membrane. DNAs were injected native, as well as marked with a fluorescent dye. The resulting membrane topology transformations were monitored in phase contrast, while DNA distribution was followed in fluorescence. We observed DNA-induced endocytosis due to the DNA/lipid membrane local interactions and complex formation. A characteristic minimum concentration (C_{endo}) of D-*erythro*-sphingosine (Sph^+) in the GUV membrane was necessary for the endocytic phenomenon to occur. Below C_{endo}, only lateral adhesions between neighboring vesicles were observed upon DNA local addition. C_{endo} depends on the type of zwitterionic (phosphocholine) lipid used, being about 10 mol% for DPhPC/Sph^+ GUVs and about 20 mol% for SOPC/Sph^+ or eggPC/Sph^+ GUVs. The characteristic sizes and shapes of the resulting endosomes depend on the kind of DNA, and initial GUV membrane tension. When the fluorescent DNA marker dye was injected after the DNA/lipid local interaction and complex formation, no fluorescence was detected. This observation could be explained if one assumes that the DNA is protected by lipids in the DNA/lipid complex, thereby inaccessible for the dye molecules. We suggest a possible mechanism for DNA/lipid membrane interaction involving DNA encapsulation within an inverted micelle included in the lipid membrane. Our model observations could help in understanding events associated with the interaction of DNA with biological membranes, as well as cationic liposomes/DNA complex formation in gene transfer processes.

Key words DNA interactions · Cationic giant unilamellar vesicle · Microinjection · Sphingosine · Endocytosis

Abbreviations *DNA 21b* Oligonucleotide DNA ss 21b · *DNA 250 bp* Calf thymus DNA ds 250 bp · *GUV(s)* Giant unilamellar vesicle(s) · *DPhPC* 1,2-Diphytanoyl-*sn*-glycero-3-phosphocholine · *DMPC* 1,2-Dimyrisoyl-*sn*-glycero-3-phosphocholine · *SOPC* β-Stearoylγ-oleoyl-L-α-phosphocholine · *eggPC* Egg phosphocholine · *PC* Phosphocholine · *Sph^+* D-*erythro*-Sphingosine · *PEG* Poly(ethylene glycol)

Introduction

Associaton of DNA with membranes and membrane/DNA complex formation play important roles in biological processes such as DNA replication and segregation (Firshein 1989). Moreover, sphingolipids have been found in nuclear membranes and chromatin fractions, and their amount seems to vary between active and repressed chromatin (Alessenko et al. 1982). The transfer of sphingolipids from sites of synthesis to the plasma membrane is blocked during mitosis, whereas that of phosphatidylethanolamine remains unchanged (Kobayashi and Pagano 1989). Sphingosine, a breakdown product of cellular sphingomyelin, is a bioactive molecule which regulates the transcription and replication processes, cell growth, differentiation, and apoptosis by protein kinase C-independent pathways (Hong et al. 1990; Zhang et al. 1990; Sakakura et al. 1996).

The binding of DNA to liposomes which contain sphingosine has been systematically investigated by differential scanning calorimetry (DSC) and fluorimetry (resonance energy transfer, RET) in liposome suspensions, and using the monolayer technique (Kinunnen et al. 1993; Kõiv and Kinnunen 1994; Kõiv et al. 1994, 1995). These experiments showed that the interaction between DNA and

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sphingosine-containing liposomes is electrostatic in nature. More specifically, this interaction should involve the protonated amino group of D-*erythro*-sphingosine (Sph^+) and the negatively charged phosphates of DNA. Binding of DNA to DMPC vesicles containing Sph^+ was dependent on the pH: the decrease of fluorescence intensity, due to RET between marked DNA and lipids, was 80% at pH 5.9, 70% at pH 7.4, and 40% at pH 8.5. DNA binding to membranes containing Sph^+ was stronger at acidic pH but was present at basic pH as well. Measurements of pK_a for Sph^+ in mixed micelles with Triton X-100 have yielded different values, i.e., 6.7 and 7.7, but the exact value in liposomes is not known. It is generally assumed that Sph^+ is at least partially protonated at physiological pH. DNA binding to liposomes containing Sph^+ could be reversed by including phosphatidic acid in the lipid membrane. Sphingosine phase separation and the formation of domains enriched in sphingosine, caused by the attached DNA, were evident from DSC and monolayer experiments.

Liposomes containing synthetic cationic lipids are currently used as carriers of antisense oligonucleotides and plasmid DNA, which regulate specific gene transfer (Felgner et al. 1987; Singhal and Huang 1994; Zelphati and Szoka 1996). Recently, liposomes containing sphingosine were used for DNA transfection with high efficiency and low toxicity (Paukku et al. 1997). We have recently shown, by using the patch clamp method of bilayers, that the electrotransfer of plasmid DNA through the membrane is dependent upon the presence of sphingosine (Hristova et al. 1997).

Considerable experimental and theoretical efforts have been focused on characterizing the structure of DNA/cationic liposome complexes (Gershon et al. 1993; Sternberg et al. 1994; Dan 1996; May and Ben-Shaul 1997). Despite the extensive studies, the mechanism of DNA ineraction with positively charged liposomes and the structure of the resulting complexes are still poorly understood. Surprisingly, until now, all studies on DNA/liposome interactions have been done on liposome suspensions. Adding DNA to such suspensions leads to liposome aggregate formation (Gershon et al. 1993; Kinunnen et al. 1993; Jääskeläinen et al. 1994; Sternberg et al. 1994; Rädler et al. 1997). No information about the effects of DNA interacting locally, as a result of local and temporal delivery of DNA to a part of the membrane of an individual vesicle, is available. This type of study would be of particular interest in modeling biological events in living cells.

Currently, we are developing a novel approach for direct optical microscopy visualization and studying the interactions of individual vesicles with colloidal particles (Angelova et al. 1994; Dietrich et al. 1997), as well as the effects of active substances injected locally by a micropipette to a part of the vesicle membrane (Wick et al. 1996). The giant unilamellar vesicles, big enough (50–150 µm in diameter) for the membrane shape and morphology transformation to be clearly seen under an optical microscope, were prepared by the liposome electroformation method. Electroformation can quickly supply a large number of giant unilamellar vesicles (GUVs), and can be used efficiently in studies involving individual vesicles, micromanipulation, and microinjection (Guedeau-Boudeville et al. 1995; Mathivet et al. 1996; Wick and Luisi 1996; Menger and Angelova 1998).

In this work we present experiments from viewing with an optical microscope the effects of short DNA microinjected locally to GUVs containing sphingosine. We observed DNA-induced endocytosis due to the local DNA/lipid membrane interactions and complex formation. We are suggesting a possible mechanism for DNA/lipid membrane interaction, complex formation, and membrane shape and morphology transformations.

Materials and methods

GUVs were prepared from 1,2-diphytanoyl-*sn*-glycero-3-phosphocholine (DPhPC). β-stearoyl-γ-oleoyl-L-α-phosphocholine (SOPC), egg phosphocholine (eggPC), and their mixtures with sphingosine at PC/Sph^+ ratios of 97:3, 93:7, 85:15, 75:25, and 67:33 mol%. (Synthetic DPhPC, Avanti Polar Lipids; synthetic SOPC, Sigma Sph^+, from bovine brain sphingomyelin, Sigma). The initial lipid deposit and the resulting GUVs become unstable for Sph^+ concentrations higher than 33 mol%. GUVs were formed by the liposome electroformation method (Angelova and Dimitrov 1986; Dimitrov and Angelova 1988).

The particular electroformation protocol established in this work was as follows: SOPC, SOPC/Sph^+, eggPC, eggPC/Sph^+ solutions were prepared in diethyl ether/methanol (9:1) at 0.3 mg/ml of total lipid; DPhPC and DPhPC/Sph^+ solutions, in chloroform/diethyl ether/methanol (2:7:1) at 0.9 mg/ml of total lipid. A droplet of lipid solution (1 µl) was deposited (avoiding sliding) on each of the two parallel platinum wires (diameter 0.8 mm, distance between axes 3 mm) and dried under nitrogen for 30 min. An a.c. electrical field, 10 Hz, 0.3 V pp, was applied to the electrodes. Distilled water (1.2 ml, pH 5.5–6.0, conductivity 3.3 µS cm^{-1}) was added (avoiding agitation) to the working chamber. The voltage was gradually increased (over 15 min) up to 2.5 V pp. The GUVs were ready in 2 h for further utilization. In each preparation at least 10 GUVs of diameter 100–150 µm were available.

The "short" DNAs used were: (1) oligonucleotide 21b (ssDNA; 21 bases: 5′-CAACCATATCTACACAGGGTC-3′) MW = 6.23×10^3 (kindly supplied by the Institute of Molecular Biology, BAS, Sofia) and (2) calf thymus 250 bp DNA (dsDNA 250 bp), MW = 1.65×10^5, prepared by ultrasonication from calf thymus highly polymerized DNA (Sigma). Molecular size was checked by 3% agarose gel electrophoresis. The DNA for local GUV microinjection was in a distilled water solution at a concentration of 0.01 mg/ml. DNA/GUV membrane interaction at a single microinjection occurred under conditions of large lipid excess with respect to the DNA (about 10^3 lipid molecules/nucleotide). DNAs were marked for fluorescent microscopy visualization with Hoechst 33258 dye (Molecular Probes, Ex/Em = 352/461 nm) at a dye/nucleotide ratio of 1:10. The Hoechst dye molecules in water solution